Palmitoylation of MICA, a ligand for NKG2D, mediates its recruitment to membrane microdomains and promotes its shedding

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Abstract

MICA and MICB (MHC-class-I-related Chain A/B) are transmembrane proteins expressed in pathological conditions that are ligands for NKG2D, an activating receptor found on cytotoxic lymphocytes. The recognition on target cells of NKG2D-ligands leads to the activation of lysis and cytokine secretion by NK cells and T cells. Besides being expressed at the cell surface, MICA/B proteins can be released as soluble proteins. Soluble NKG2D-ligands downmodulate expression of the NKG2D receptor on lymphocytes, leading to a diminished cytotoxic response. Prior studies suggested that recruitment of MICA/B molecules to cholesterol-enriched microdomains was an important factor regulating the proteolytic release of these molecules. We now show that recruitment of MICA to these microdomains depends on palmitoylation of two cysteine residues that allow MICA molecules to reside in the membrane in the same domains as caveolin-1. Compared with wild-type molecules, non-palmitoylated mutant MICA molecules were shed to the supernatant with low efficiency; however, both wild-type and mutant MICA were able to trigger NK cell cytotoxicity. These data suggest that the presence of NKG2D-ligands at the plasma membrane is sufficient to activate cytotoxicity and reflect the need of different ligands to exploit different cellular pathways to reach the cell surface upon different stress situations.

Introduction

MICA and MICB proteins are a family of polymorphic genes mapping within the MHC region, from which they receive their name, MHC class I-related Chain A and B [1]. However, in contrast to MHC class I molecules, MICA/B do not associate with β2-microglobulin or present antigenic peptides, nor are they expressed constitutively. Instead their expression is induced on many types of tumour cells and after infection by viruses or intracellular bacteria [2]. They bind the activating immune receptor NKG2D, constitutively expressed on all NK cells as well as $CD8^+$ αβ T cells and γδ T cells [3]. The recognition by NKG2D of its ligands expressed on target cells induces lysis and cytokine secretion by NK cells and T cells. However, MICA/B proteins can also be shed from cells and interaction with these soluble proteins leads to loss of cell surface NKG2D receptor and lymphocyte unresponsiveness [2, 4]. Indeed, the presence of high levels of soluble NKG2D-ligands in patient serum is a marker for poor prognosis in various types of cancer [5-7]. Thus it seems clear that the regulation of expression and shedding of MICA/B molecules plays an important role in the regulation of the function of the cytotoxic lymphocytes of the immune system [8].

Previous studies have shown that proteolytic cleavage of MICA/B depends on metalloproteases [9, 10], specifically, the ADAM (A Disintegrin And Metalloproteinase) family members, ADAM9, ADAM10 and ADAM17 (also known as TACE, TNF-Alpha-Converting Enzyme) [11-13] and the matrix metalloprotease MMP14 [14]. However, these metalloproteases are all rather widely expressed suggesting that there may be other levels of regulation of MICA/B shedding. The thiol isomerase ERp5 is another protein that has been reported to influence the shedding of MICA molecules [15]. The recruitment of MICB molecules into cholesterol and sphingolipid enriched microdomains (detergent-resistant membranes, DRMs) has also been suggested to aid efficient proteolytic release of these molecules [12], as treatment of cells with an inhibitor of palmitoylation was shown to block the recruitment of MICB molecules to DRMs and to reduce the shedding of MICB molecules. However, while treatment with the palmitate analog 2-bromo-palmitate has been widely used to inhibit the palmitoylation of proteins [16], this is not a particularly selective agent, moreover, the mechanism by which it inhibits palmitoylation is unknown [17]. Palmitoylation refers to the addition of a 16-carbon fatty acid by thioester linkage to cysteine residues, although proteins can be modified by a variety of fatty acids with different chain lengths and insaturations. For this reason, the term palmitoylation is used in a general manner of referring to S-acylation. It has been suggested that S-acylation preferentially targets molecules possessing transmembrane domains to specialised microdomains of the cell membranes enriched in cholesterol, DRMs, also called lipid rafts [18]. MICA and MICB contain two conserved juxtatransmembrane cysteines which are putative sites for

palmitoylation. In this paper, a MICA molecule, where these cysteines have been mutated to serines, is shown to be defective in palmitoylation and recruitment to DRMs. Using this MICA palmitoylation mutant we have studied the functional role of this modification for both DRM localisation and MICA function. In particular, the shedding of soluble MIC molecules and immune recognition by NK cells were analysed.

Results

MICA can be palmitoylated on cysteine residues in the cytoplasmic tail

We have reported that the recruitment of MIC to DRMs was important for efficient shedding of these molecules [12]. Here we have investigated the molecular basis governing the association of MIC to these cholesterol and sphingolipid enriched domains. Previously, treatment of cells with 2-bromo-palmitate, an inhibitor of protein palmitoylation, was shown to markedly reduce both shedding of soluble MIC molecules and the recruitment of MICA/B to DRMs, suggesting that palmitoylation might be a prerequisite for recruitment of MICA/B proteins to cholesterol enriched microdomains, could be palmitoylation. Both MICA and MICB contain two cysteines at the cytoplasmic tail (Figure 1A) that are putative sites of palmitoylation. Thus the relevance of these cysteines for recruitment of MICA into cholesterol-enriched membrane domains was investigated by mutagenesis: the Cys residues in position 306 and 307 were mutated into serines (MICA2CS) (Figure 1A). Both the MICA wild type and MICA2CS palmitoylation mutant molecules were stably expressed in CHO cells (CHO-MICA and CHO-MICA2CS) and similar cell surface expression of the wild-type and mutant molecules was observed by flow cytometry (Figure 1B). Next, a non-radioactive labelling method based on the substitution of the metabolically labelled fatty acid by a biotinylated probe [19, 20] was used to investigate the level of palmitoylation of the wild-type and the mutant MICA molecules. CHO cells transfected with MICA or MICA2CS were labelled with ω-azido pentadecanoic acid and lysed. After immunoprecipitation and click chemistry labeling, fatty acid-modified proteins became biotinylated and could be visualised on western blot analysis with streptavidin-HRP (Figure 1C). Palmitoylated MICA was observed for the wild-type transfectant, however mutation of the cysteine residues led to the absence of detectable palmitoylation of MICA molecules. The positive control protein caveolin-1 was shown to be palmitoylated in both preparations and equal amounts of MICA molecules were present in the lysates. Thus, this experiment confirmed a previous report indicating that MICA molecules could be palmitoylated [21] and demonstrated that the paired cysteines 306 and 307 of the cytoplasmic tail are necessary for palmitoylation of MICA.

Palmitoylation of MICA is relevant for its association with membrane microdomains enriched in cholesterol (DRMs)

It is known that palmitoylation may affect the trafficking and stability of proteins [22]. However, pulse-chase experiments showed that the EndoH resistant band that corresponds to the mature form of the protein appears in both MICA and MICA2CS with similar kinetics confirming that the maturation rate of MICA was not altered by the mutation of the Cys residues in the cytoplasmic tail (*data not shown*). Next, since covalent modification with

palmitic acid has been shown to be important for the association of various transmembrane proteins with DRMs, we studied whether palmitoylation was part of the molecular basis for the recruitment of MICA to DRMs. For these experiments, a well established biochemical approach based on the solubilization of membranes using non-ionic detergents, followed by fractionation in sucrose gradients [23] was used. To assess the efficiency of fractionation, the protein caveolin-1 was included as a marker of the fractionation. In our CHO transfectant system, although the majority of MICA proteins were present in the soluble fractions, a proportion of these molecules was also present in caveolin-1 positive fractions, suggesting that MICA could be recruited to DRMs (Figure 2A). When the MICA2CS palmitoylation mutant was analysed, no MICA molecules were found in the DRM fractions. These data demonstrate that palmitoylation of these juxtamembrane Cys residues in the cytoplasmic tail is essential for the MICA partition into DRM microdomains.

The presence of MICA in DRMs poses the question whether they would be also included in the specialized invaginations of the membrane known as caveolae. These are quite stable, specialised membrane invaginations that under certain circumstances can become internalized [24]. To investigate the presence of MICA in caveolae, co-immunoprecipitation experiments of MIC and caveolin-1 were performed. Caveolin-1 was immunoprecipitated from CHO cells expressing either MICA or MICA2CS, run on SDS-PAGE and then analysed by western blot with antibody directed to MICA/B (Figure 2C). These data indicate the inclusion of MICA and caveolin-1 in the same detergent micelles and, thus, in the same membrane microdomains, however direct interaction between the two proteins was not demonstrated in these experiments. The absence of Cys in the cytoplasmic tail of MICA2CS led to the loss of MIC in caveolin-1-containing domains. Thus, MICA requires the Cys residues present in their cytoplasmic tail for both recruitment to DRMs and also for its presence in domains rich in caveolin-1.

A Cys motif in the cytoplasmic tail of MICA is necessary for efficient shedding

The above experiments established that mutation of the juxtamembrane cysteine residues abolished palmitoylation and recruitment of MICA to DRMs/caveolae. Thus, it was of interest to analyse the significance of the inclusion of MICA in caveolae or DRMs for the biology of this molecule, in particular, for shedding and for recognition by NK cells. We have recently reported that the recruitment of MICA/B to DRMs was important for efficient shedding and that treatment of cells with inhibitors of palmitoylation caused a decrease in release of soluble MIC [12]. However treatment with 2-bromo-palmitate is likely to affect a wide range of cellular processes and proteins hence, we characterized the effect of the elimination of palmitoylation of the cytoplasmic tail of MICA on proteolytic release of this molecule. The release of soluble MICA from CHO cells transfected with either MICA or MICA2CS was

evaluated by sandwich ELISA in tissue culture supernatants collected at various time points (Figure 3A). The shedding of MICA2CS, compared to MICA, was considerably reduced at all the time points although, with time, accumulation of the palmitoylation mutant could be observed in the supernatant. This indicates that palmitoylation promotes shedding but that release can still occur outside DRMs.

This decreased shedding of the MICA2CS protein was also seen in U373 transfectants demonstrating that this effect did not depend on the identity of the transfected cell (Supporting Information Figure 1). Moreover, the residual level of shedding from the MICA2CS transfectant was largely independent of the activity of several proteases, except for metalloproteases (Figure 3B). The inhibition of MICA shedding seen after treatment with the broad spectrum metalloprotease inhibitor BB94 was statistically significant ($p<0.05$) whereas no other inhibitor significantly changed the shedding of MICA2CS. These findings support the idea that palmitoylation of MICA and recruitment to DRMs is important for efficient proteolytic release of soluble MICA molecules and suggest that the decreased shedding of MICA/B observed on treatment with inhibitors of palmitoylation was due to blockade of recruitment of MICA/B to DRMs rather than any effect of the inhibitor on ADAM17/TACE [12].

The absence of Cys motifs in MICA does not affect NK cell cytotoxicity

To evaluate the effects of MICA palmitoylation for NK cell recognition, the abilities of the MICA wild-type and A2CS transfectants to activate cytotoxicity were compared using freshly isolated (resting) NK cells. Both MICA and MICA2CS transfected cells were equally susceptible to NK cell cytotoxic attack (Figure 4A, Supporting Information Figure 2). Given that released, soluble MICA can inactivate NK cell responses and that there are clear differences in the efficiency of shedding between MICA and MICA2CS NK cell cytotoxicity was also investigated after blocking MICA shedding by treatment with metalloprotease inhibitors. However, even when shedding was blocked by treatment with the metalloprotease inhibitor GM6001, no difference between NK cell cytolytic activity against MICA and MICA2CS was observed (Figure 4B, Supporting Information Figure 2). Cytotoxicity assays were also performed using NK cell lines, activated by culture in the presence of IL-2, and again NK cells lysed CHO cells expressing MICA or MICA2CS equally well (Figure 5A, Supporting Information Figure 3). It has been suggested that the threshold of stimulation needed to trigger cytotoxicity and IFN-γ production are different [25], however the MICA and MICA2CS transfectants were equally able to stimulate production of IFN-γ by polyclonal NK cell lines maintained in IL-2 (Figure 5B, Supporting Information Figure 3). Therefore, overall,

these results suggest that the addition of palmitic acid to MICA and the recruitment of palmitoylated MICA to DRMs are not contributing to target cell recognition by NK cells.

Discussion

The data presented in this paper demonstrate that palmitoylation of the cysteine residues present in the cytoplasmic tail of MICA is a key modification for its recruitment into DRM microdomains, since a mutant MICA molecule, lacking the two conserved cysteines, was absent from these specialised membrane microdomains. The proximity between MICA and caveolin-1, the main component of caveolae, was also demonstrated. Assessment of the functional implications of palmitoylation on MICA revealed that the loss of the two conserved cysteines in the cytoplasmic tail was associated with a decrease in the proteolytic release of MICA. In contrast, the lack of palmitoylation of MICA, and the consequent absence of this molecule in cholesterol and sphingolipid enriched domains at the plasma membrane does not seem to have a major impact on NK cell recognition and cytotoxic activity mediated by the NKG2D receptor.

The data presented here suggest that the recruitment of MICA into specialised microdomains has a major significance in the release of these ligands as soluble molecules. The presence of soluble NKG2D-ligands can affect the function of NK cell and T cell effector immune cells as has been demonstrated both *in vitro* and *in vivo* using animal models [4, 26, 27]. These observations are consistent with previous data showing that although only a relatively small proportion of MICA is found in DRMs, the presence of MIC molecules in these microdomains is critical for efficient proteolytic release of soluble MIC molecules [12]. In fact, published data showed that treatment of cells with inhibitors of palmitoylation reduces the release of soluble MICB molecules and treatment with broad spectrum inhibitors of metalloproteases increases the fraction of MICB molecules found in DRMs. Here, we used a cleaner, more specific, system to eliminate palmitoylation by mutation of cysteine residues, and demonstrated that this modification is indeed responsible for the inclusion of MICA in DRMs and participates in shedding of the molecule.

Only a proportion of the MICA proteins are palmitoylated within the cell and this is not surprising since it is known that several proteins can undergo successive cycles of palmitoylation and depalmitoylation mediated by palmitoyl acyl transferases, adding palmitic acid molecules, which can be removed by cytosolic acyl-protein thioesterases [28]. Since protein palmitoylation is a reversible modification that can be dynamically regulated by extracellular stimuli [29], it would be interesting to investigate whether MICA palmitoylation can be modulated by cellular stress such as infection or tumour transformation, thus influencing the localisation of MICA molecules in the cholesterol enriched microdomains of the membranes, and so MICA shedding. However, the biological significance of protein

palmitoylation of transmembrane molecules includes more than just recruitment to DRMs. As happens with other proteins, palmitoylation of MICA could also affect the trafficking, endocytosis and stability of this molecule. For example, it is known that palmitoylation can protect proteins from ubiquitylation and degradation [30]. Virally-induced ubiquitylation of MICA has been reported [31] and it is interesting to note that the cysteine substrates for palmitoylation are adjacent to the lysine residues of MICA that are ubiquitylated by the Kaposi's sarcoma-associated herpesvirus ubiquitin ligase [31]. In other systems, protein de-palmitoylation followed by ubiquitination promotes internalisation and sorting to intracellular compartments such as multivesicular bodies (MVB) that ultimately fuse with lysosomes or with the plasma membrane to release exosomes [32]. These observations reinforce the idea that palmitoylation, ubiquitylation and endocytosis of MICA are important aspects of the cell biology of these molecules that could happen either sequentially or in cycles and this needs further investigation. In this context it is interesting to note that MICA co-immunoprecipitated with caveolin-1, while a mutant MICA lacking the two cysteines in the cytoplasmic tail did not co-precipitate with caveolin-1. These data suggest that MICA is found in the same membrane microdomains as caveolin-1 and, given the clear association of the predominantly caveolae-associated protein caveolin-1 with endocytosis [33], pose the question of whether the association with caveolin is relevant for clathrin-independent endocytosis of MICA. Similarly, the observation that the localization of the matrix metalloproteinase MT1-MMP to caveolin-enriched domains has an important effect on the control of its enzymatic activity [34, 35], raises the question of whether the presence of MICA in caveolae is relevant for shedding.

The NKG2D receptor has been shown to localise to cholesterol enriched domains upon NK cell activation [36]. However, the relevance of this recruitment of both receptor and ligands to DRMs during the NK cell-target cell interaction is unclear. Here we demonstrate that the lack of two cysteine residues at the beginning of the cytoplasmic tail of MICA does not significantly affect the ability of these mutant molecules to stimulate cytotoxic activity or IFN-γ production by either resting or IL-2 activated NK cells. These results are consistent with our recent study, showing that a naturally occurring transmembrane form of ULBP2 was as capable of enhancing NK cell activation as the GPI-linked form of ULBP2 [37]. In contrast, Martinez et al. [38] have observed that redistribution of ULBP1 outside of DRM, through the replacement of the GPI linkage in ULBP1 by a transmembrane region of CD45, resulted in diminished NK cell responses to P815 cells expressing these molecules. The specific contribution of various transmembrane regions to the distribution and properties of receptor ligands on target cells has yet to be explored. Our data differ also from the observations of Eleme *et al.* who reported that a truncated form of MICA lacking the complete cytoplasmic tail, including the putative site for S-acylation, was expressed at the cell surface but was unable to overcome inhibition of NK cell cytotoxicity, as wild-type MICA did [21]. However, an

important difference between those experiments and ours is that the mutant MICA molecule studied by Eleme *et al.* lacked the whole cytoplasmic tail, suggesting that some other region or modification of the cytoplasmic tail of MICA might be important for the regulation of NK cell cytotoxicity.

One important question in the field of NKG2D is why a single receptor has such a variety of different ligands, including a large number of alleles for MICA/B and two families of proteins with different transmembrane attachments. Since all of them seem to be able to recognise NKG2D receptor, variability seems to be important for regulation of the surface expression and release of NKG2D-ligands. Variation thus could play an important role in the adaptation of the cell to different stress situations and/or pathogen infection. The data presented here support the idea that modifications at the C-terminal region of NKG2D-ligands affects important aspects of the biology of these proteins such as inclusion in membrane microdomains and shedding, but not receptor-ligand recognition.

Materials and Methods

Site directed mutagenesis

A construct encoding a MIC molecule where cysteines 306 and 307 in the cytoplasmic tail were mutated to serine residues (MICA2CS) was prepared by PCR (Quikchange mutagenesis kit, Stratagene), using a plasmid containing the MICA*019 allele as template [39] and the oligonucleotides (Sigma-Genosys Ltd): 5'TTC TAT GTC CGT TcT TcT AAG AAG AAA ACA TCA GC3' and 5'GC TGA TGT TTT CTT CTT AgA AgA ACG GAC ATA GAA3' (lower case indicates the nucleotides mutated). The integrity of the MICA2CS plasmid construct was verified by DNA sequencing analysis. A schematic representation of the wild-type and mutant constructs is depicted in Figure 1A.

Cells

Chinese Hamster Ovary cells (CHO) were maintained in Hams F12 medium supplemented with 10% fetal calf serum. For transfection, the expression plasmids encoding either MICA*019 or MICA2CS were mixed (9:1 ratio) with a vector conferring resistance to puromycin [40], then CHO cells were transfected with this mixture using Lipofectamine 2000. Stable transfectants were prepared by culture of transfected CHO cells in selective medium (8 μg/ml puromycin, Calbiochem) and, where necessary, by cell sorting. U373-MICA and MICA2CS cells were produced by transfecting the glioma cell line U373 (a gift of Dr J.H. Sinclair, Department of Medicine, University of Cambridge) with pcDNA3 plasmids containing the constructs as in ref. 39.

Human NK cells (95 – 99% $CD3^-$ $CD56^+$) were freshly isolated from peripheral blood using a negative selection kit (Miltenyi Biotec), resuspended in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with 10% human serum (Valley Biomedical) without IL-2 or feeders. NK cells were used for experiments within two days ("resting NK cells").

IL-2 activated NK cells were expanded in media containing recombinant IL-2 and autologous irradiated PBL as feeders. IL-2-activated NK cells were used between 2 or 3 weeks after isolation, and 3-5 days post-IL-2 stimulation. The NK cells were washed with IL-2 free media prior to their use in assays.

Antibodies and reagents

Anti-MICA/B monoclonal and polyclonal antibodies were purchased from R&D systems (FAB13001, monoclonal anti-MICA/B and AF1599, polyclonal anti-MICA/B), Immatics (AMO1, MICA-specific) and Santa Cruz Biotechnology (6D4, anti-MICA/B). Anti-

MICA-specific antibodies were from R&D system (anti-MICA biotin conjugated, BAF1300) and Immatics (AMO1, MICA-specific). Isotype control mouse antibodies and control rabbit serum were from Sigma-Aldrich. Rabbit anti-caveolin-1 antibody was from BD Transduction Laboratories. Streptavidin-HRP was purchased from GE Healthcare. The metalloprotease inhibitors GM6001 (Chemicon) and BB94 (Tocris Bioscience) were used at final concentrations of 25 μM and 10 μM respectively. Leupeptin and Pepstatin A (Sigma) were used at a final concentration of 1 μg/ml.

Flow cytometry

1 × 10^6 cells were recovered in PBS containing 2% bovine serum albumin and incubated with anti-MICA/B mouse Abs (R&D systems) followed by FITC-labelled F(ab')2 fragments of goat anti-mouse Ig (Dako). Samples were analysed using a FACScan II flow cytometer (Becton Dickinson). Dead cells were excluded from all the analysis by staining with 1 μg/ml propidium iodide (Sigma). Samples were analyzed in duplicates (10^5 cells per sample) using Cell Quest Software to measure Mean Fluorescence Intensity (MFI) of FITC molecules.

DRMs fractionation

Detergent resistant membranes (DRMs) isolation was performed as described previously [12]. Briefly, 20 x 10^6 CHO-MICA and CHO-MICA2CS cells were lysed in TNE buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% Brij-58 (Sigma) and protease inhibitors (1 μM leupeptin and 1 μM pepstatin). Lysates were homogenised with 10 strokes with a Dounce homogeniser, diluted with an equal volume of 85% sucrose in TNE and then placed at the bottom of clear ultra centrifuge tubes. A discontinuous sucrose gradient was overlaid on top of this suspension (6 ml of 30% sucrose and 4 ml of 5% sucrose, both in TNE). Samples were centrifuged at 200,000 g at 4°C for 20 hours. 1 ml fractions were collected from the top to bottom, and deoxycholate (DOC) was added to a final concentration of 0.2%. Samples were analysed by SDS-PAGE and western blot.

Western blot

Samples were analysed on 12% SDS-PAGE gels and transferred to Immobilon-P (Millipore). Membranes were blocked using 5% non-fat dry milk in PBS containing 0.1% Tween-20 and incubated with goat polyclonal biotinylated anti-MICA/B antibody (R&D systems) or anti-caveolin-1 (BD Transduction Laboratories) for 1 hour, followed by a horseradish peroxidase-conjugated secondary reagent. Proteins were visualized using the ECL system (GE Healthcare).

Detection of palmitoylated proteins

Protein palmitoylation was analysed using a novel, non-radioactive approach [19, 20, 41]. This procedure is based on the metabolic labelling of cells with a palmitic acid analogue and click chemistry. CHO-MICA and CHO-MICA2CS cells stably transfected with MICA or MICA2CS were washed with PBS and pre-treated with 10 μM GM6001 in serum free Hams-F12 medium during 30 minutes to minimise the loss of MICA molecules by shedding. Cells were then metabolically labelled by adding 100 μM ω-azido pentadecanoic acid for 2 hours at 37ºC in 5% CO_2. Cells were washed in PBS, harvested and lysed in buffer containing 50 mM triethanolamine pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, 1 μM leupeptin and 1 μM pepstatin for 30 min at 4ºC. Post-nuclear lysates were obtained by centrifugation at 20,000 g. Lysates were precleared with Pansorbin (Merk) and immunoprecipitated with either anti-MICA antibodies (AMO1 and 6D4). Caveolin-1 protein was used as a positive control. Immunoprecipitated proteins were recovered with Protein G sepharose beads. Beads were resuspended in lysis buffer containing 4% SDS, and fatty acid labelled proteins were biotinylated by chemoselective reaction with 100 μM alkyne-biotin, 1 mM Tris(2-carboxyethyl)phosphine (TCEP, Pierce), 100 μM Tris-(benzyltriazolylmethyl)amine (TBTA, Sigma) and 1 mM $CuSO_4$ for 1 h at room temperature. Click reactions were terminated by the addition of SDS-PAGE loading buffer. Samples were analysed by SDS-PAGE and Western blot where streptavidin-HRP was used as detection reagent to visualise biotinylated, and hence, palmitoylated immunoprecipitated MICA and caveolin-1.

Immunoprecipitation

Cells were lysed in 50 mM Tris pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1% Brij-58. Nuclei were removed by centrifugation and lysates were precleared by incubation with Pansorbin. Anti-caveolin-1 antibodies were added to lysates, incubated for 1 hour and recovered using Protein A sepharose beads (Pharmacia). Rabbit Ig was used as a negative control. Beads were washed three times and resuspended in SDS-PAGE-loading buffer. Samples were resolved in SDS-PAGE and western blot was performed using biotinylated goat polyclonal antibodies against MICA/B (R&D systems).

ELISA

Soluble MICA was measured using a sandwich ELISA as reported in ref 12. As a coating antibody, mouse anti-MICA mAb from R&D Systems was used at 4 μg/ml in Borate Buffered Saline (BBS, 10 mM sodium borate and 150 mM NaCl pH 8.2). After blocking for 2 hours with BBS + 2% BSA, tissue culture supernatants were incubated for 1 hour at 37°C. After washing with BBS with 0.05% Tween-20, MICA proteins were detected using

biotinylated goat anti-MICA (0.4 µg/ml) antibodies and the assay was developed by incubation with streptavidin-HRP (1/2000; Amersham Biosciences) and a peroxidase substrate system (ABTS, Roche). The absorbance was measured at 410 nm with a reference wavelength of 490.

Cytotoxicity assays

NK cell cytotoxicity was evaluated by non-radioactive, cytotoxicity assays based on time-resolved fluorometry assays as described [42]. 5 x 10^6 CHO, CHO-MICA and CHO-MICA2CS cells were labelled with a fluorescent enhancing ligand (40 µM Delfiabatda, Perkin Elmer) for 30 minutes at 37°C. Cells were washed in IMDM medium with 10% fetal calf serum and 1 mM sulfinpyrazone (Sigma), to reduce spontaneous label release. Target cells were co-incubated with resting or IL-2-activated NK cells at different E:T ratios in triplicates for 2 hours at 37°C. When IL-2-activated NK cells were used, the incubation time was 4 hours. Supernatants were transferred to a europium solution (0.3 M acetic acid in a Dissociation-Enhanced Lanthanide Fluorescent Immunoassay, DELFIA solution, Perkin Elmer). Lysis of target cells was measured by fluorescence of the ligand released in supernatants, using a Wallac plate reader (Perkin Elmer). Samples were analyzed in triplicates.

Intracellular staining IFN-gamma

IL-2 activated NK cells (2 x 10^5) were added to 4 x 10^5 CHO, CHO-MICA or CHO-MICA2CS cells in 200 µL of complete medium. Cells were incubated for 1 hour at 37°C in 5% CO2. Thereafter, monensin was added to the cultures (final concentration 1µM), which were incubated for 5 more hours. After this incubation, the cells were spun down, washed once with PBS containing 2 % bovine serum albumin and stained with FITC-conjugated mAb to CD56. Thereafter, cells were washed, fixed with 2% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.5% Saponin, and stained intracellularly with PE-conjugated mAb specific for IFN-g (Biolegend, clone 4S.B3). Finally, cells were washed and the samples were analysed using a FACScan II flow cytometer (Becton Dickinson).

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Conflict of interest

The authors declare no financial of commercial conflict of interest.

Figure legends

Figure 1. Palmitoylation of MICA occurs at cysteine residues in the cytoplasmic tail.

(A) Schematic representation of the amino acid sequence of MICA transmembrane region and cytoplasmic tail. Two Cys at the beginning of the cytoplasmic tail of the WT molecule were mutated into two Ser to produce the MICA2CS mutant. The putative transmembrane domain is boxed.

(B) Cell surface expression of both MICA and MICA palmitoylation mutant (MICA2CS) in stably transfected cells. CHO cells were stably transfected with MICA wild type and MICA palmitoylation mutant (MICA2CS). Cell surface expression was assessed by flow cytometry. The black and grey shaded histograms show isotype control staining for the MICA and MICA2CS transfectants respectively.

(C) Palmitoylation of MICA requires the presence of cysteines in the cytoplasmic tail. CHO cells stably transfected with either MICA or MICA2CS (A2CS) were labelled with the 15-carbon ω-azido fatty acid (synthetic analogue for fatty acylation). After preparing lysates, acyl groups were exchanged with alkyne biotin, resulting in biotinylation of the fatty acid modified proteins. Lysates were immunoprecipitated with anti-MICA antibodies and separated by SDS-PAGE. Palmitoylated proteins were detected by western blot using streptavidin-HRP (see *Materials and Methods*). As a positive control for the detection of palmitoylated proteins, lysates immunoprecipitated with anti-caveolin-1 antibody were processed in parallel (cav-1). As a loading control, the total amount of MICA in whole cell lysates in both WT and mutant cell lysates was assessed by western blot (right). Data are representative of three experiments.

Figure 2. MICA palmitoylation is relevant for its recruitment into cholesterol-enriched membrane domains.

(A) A proportion of MICA is present in cholesterol-enriched membrane domains DRMs but is absent in the palmitoylation mutant. CHO-MICA and CHO-MICA2CS transfectants were lysed in buffer containing 1% Brij-58, homogenised and fractionated by centrifugation in a discontinuous sucrose gradient for 20 hours at 200,000 × g. Fractions were collected from top to bottom and separated by SDS-PAGE. Western blot was performed using antibodies against either MICA or caveolin-1 as control for the efficiency of fractionation. Quantitation of the blots was performed using the ImageJ program (right).

(B) MICA co-immunoprecipitates with caveolin-1 only if the two Cys in the cytoplasmic tail are present.

CHO cells expressing either MICA or MICA2CS were lysed in buffer containing 1% Brij-58 and immunoprecipitated using either rabbit polyclonal antibody against caveolin-1 or control

Ig from non-immune rabbit serum. The immunoprecipitates were separated on SDS-PAGE and MICA was detected by western blot. Total lysates of CHO-MICA and CHO-MICA2CS transfectant cells were separated by SDS-PAGE and western-blotted with anti-MIC antibodies to confirm equal loading of MICA (right). Data are representative of four experiments.

Figure 3. Shedding of the palmitoylation-deficient MICA2CS molecule is decreased. (A) Tissue culture supernatant from either CHO-MICA or CHO-MICA2CS cells were harvested at 1h, 2h, 4h and 8h for analysis by sandwich ELISA as described in *Materials and Methods*. The amount of soluble MICA was calculated from the absorbance, measured at 410 nm. Data were analyzed with a Students T-test, * $p<0.05$, ** $p<0.001$. B) Tissue culture supernatant from either CHO-MICA or CHO-MICA2CS cells pre-incubated with the indicated inhibitors were harvested at 8h and analysed by sandwich ELISA. Data are presented as mean + SD of n=3 samples and are representative of five independent experiments.

Figure 4. MICA and the palmitoylation mutant MICA2CS are equally able to trigger cytotoxicity by resting NK cells.

(A) Target cells expressing either wild type MICA or MICA2CS molecules are lysed equally by resting NK cells. Human NK cells, freshly isolated from peripheral blood, (resting NK cells) were used as effector cells in europium-release killing assays (see *Materials and Methods*). Untransfected CHO cells or CHO cells stably transfected with either MICA or MICA2CS were used as target cells. Targets were labelled with the fluorescent enhancing ligand DELFIA-BATDA (Perkin Elmer), washed and co-incubated with resting NK cells at the indicated E: T ratios for 2 h at 37°C. Supernatants were transferred to a europium solution and the lysis of target cells was measured by assaying the release of fluorescent ligand.

(B) NK lysis of MICA and the palmitoylation mutant is not affected by the inhibition of metalloproteinases. CHO-MICA or MICA2CS cells were cultured alone or with the inhibitor of metalloproteinases GM6001 (25 μM) for 2 hours at 37°C before using them as target cells in europium-release killing assays performed as in (A). Data are presented as mean + SD of n=3 and are representative of four experiments.

Figure 5. MICA and the palmitoylation mutant MICA2CS are equally able to trigger cytotoxicity and cytokine production by IL-2 activated NK cells.

(A) CHO cells expressing either MICA or MICA2CS are equally efficient in triggering cytotoxicity by IL2-activated NK cells. IL2-activated NK cells were used 3 days post-IL2 stimulation in europium-release killing experiments. The incubation time was 2 h at 37°C.

(B) MICA and MICA2CS expressing CHO cells are equally efficient in triggering IFN-γ production by IL2-activated NK cells. NK cells were cultured alone or co-cultured with the indicated target cells for 6 hrs in the presence of brefeldin A. Cells were then stained with FITC-labelled CD56, fixed with 4% paraformaldehyde, permeabilised by treatment with 0.1% Saponin and stained with PE-labelled IFN-γ specific antibodies. Data are presented as mean + SD of n=3 and are representative of three experiments.

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A

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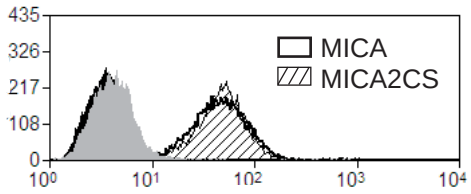
MICA 2CS: ----------SS----------....

B



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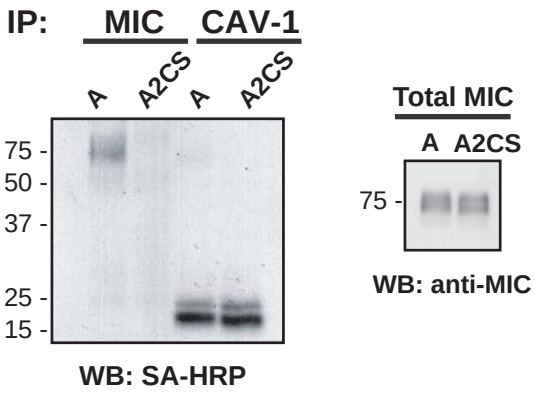
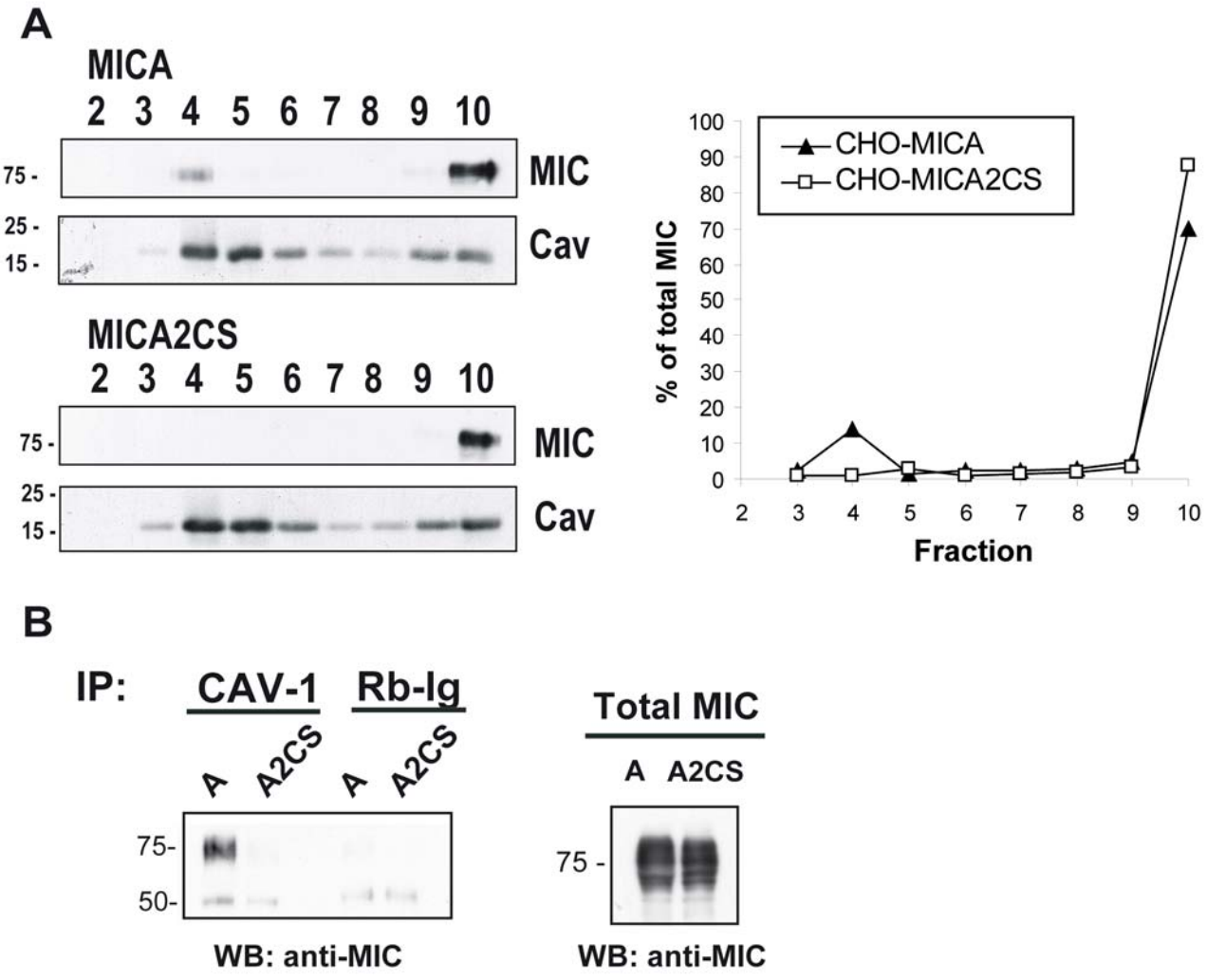


Figure 1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75 -
MIC
25 -
15 -
Cav
MICA2CS
2 3 4 5 6 7 8 9 10
75 -
MIC
25 -
15 -
Cav
CHO-MICA
CHO-MICA2CS
% of total MIC
Fraction
B
IP:
CAV-1
Rb-Ig
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75-
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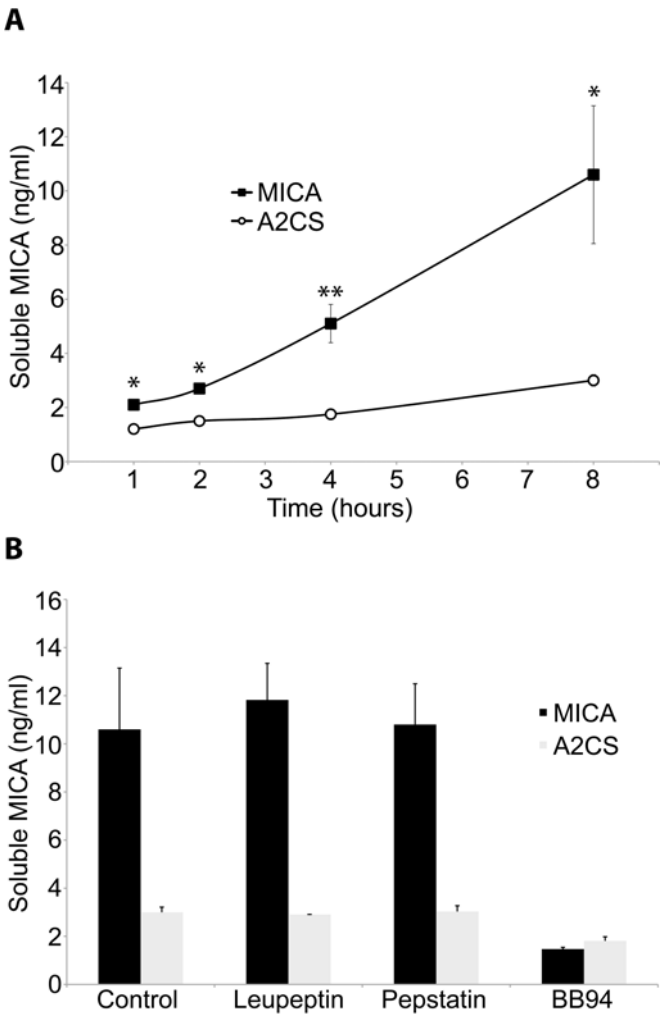
Total MIC
A
A2CS
75 -
WB: anti-MIC

Figure 2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Soluble MICA (ng/ml)
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12
10
8
6
4
2
0
MICA
A2CS
*
*
**
*
1
2
3
4
5
6
7
8
Time (hours)
B
Soluble MICA (ng/ml)
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14
12
10
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6
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2
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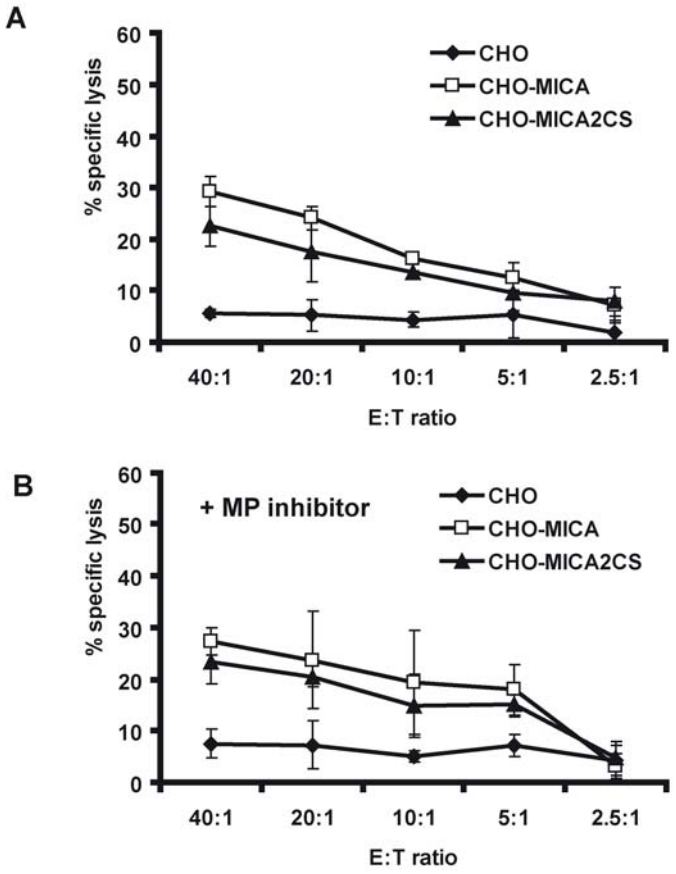
A2CS
Control
Leupeptin
Pepstatin
BB94

Figure 4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50
40
30
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CHO
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CHO-MICA2CS
40:1
20:1
10:1
5:1
2.5:1
E:T ratio
B
+ MP inhibitor
% specific lysis
60
50
40
30
20
10
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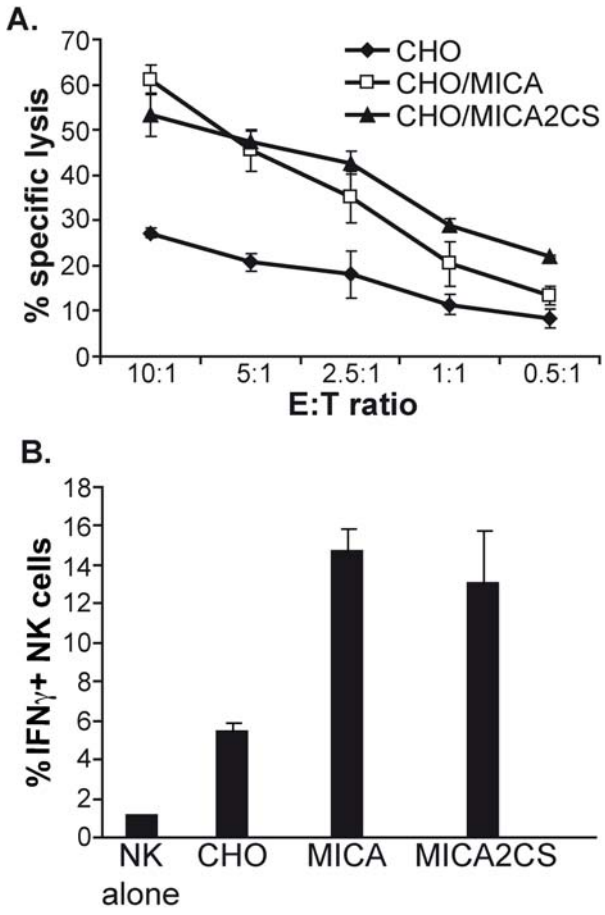
5:1
2.5:1
E:T ratio

Figure 5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CHO/MICA
CHO/MICA2CS
10:1
5:1
2.5:1
1:1
0.5:1
E:T ratio
B.
% IFNγ+ NK cells
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10
8
6
4
2
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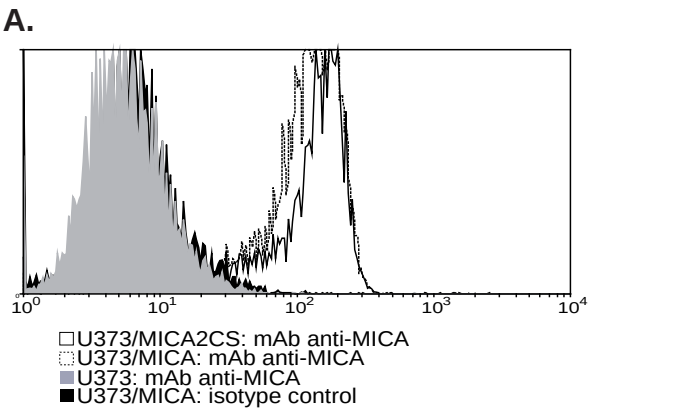
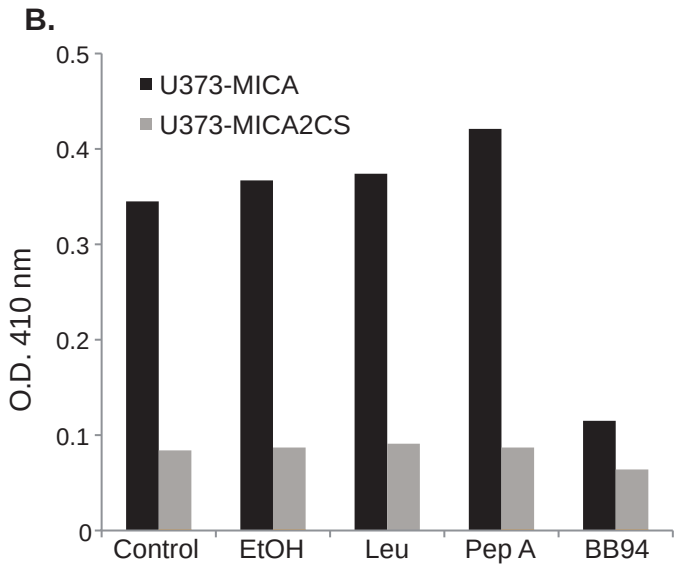
NK alone
CHO
MICA
MICA2CS

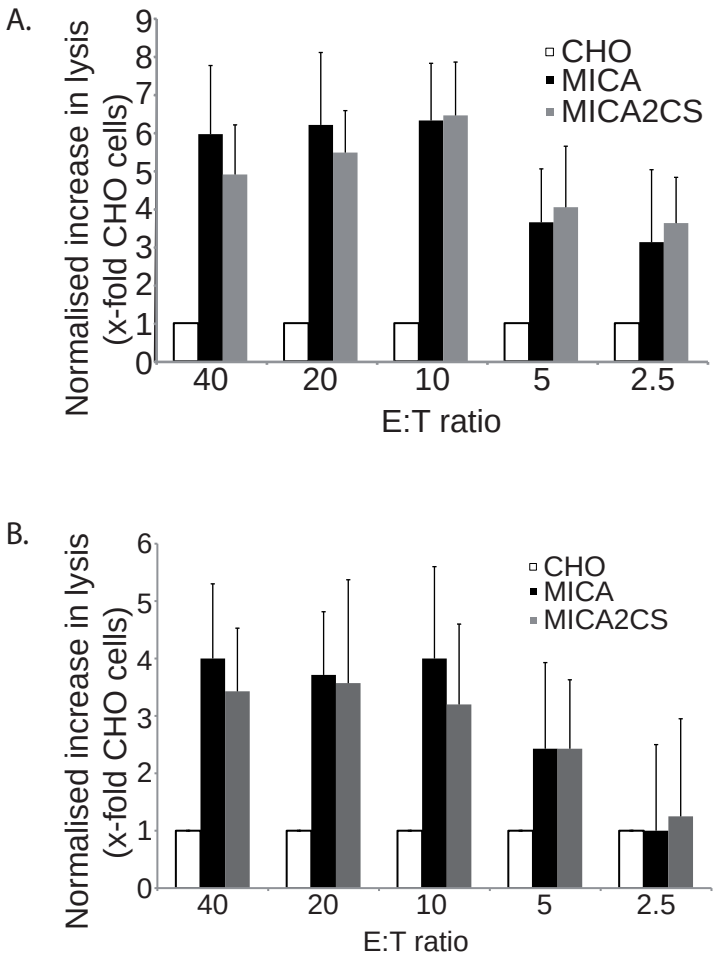
Supplementary Figure 1





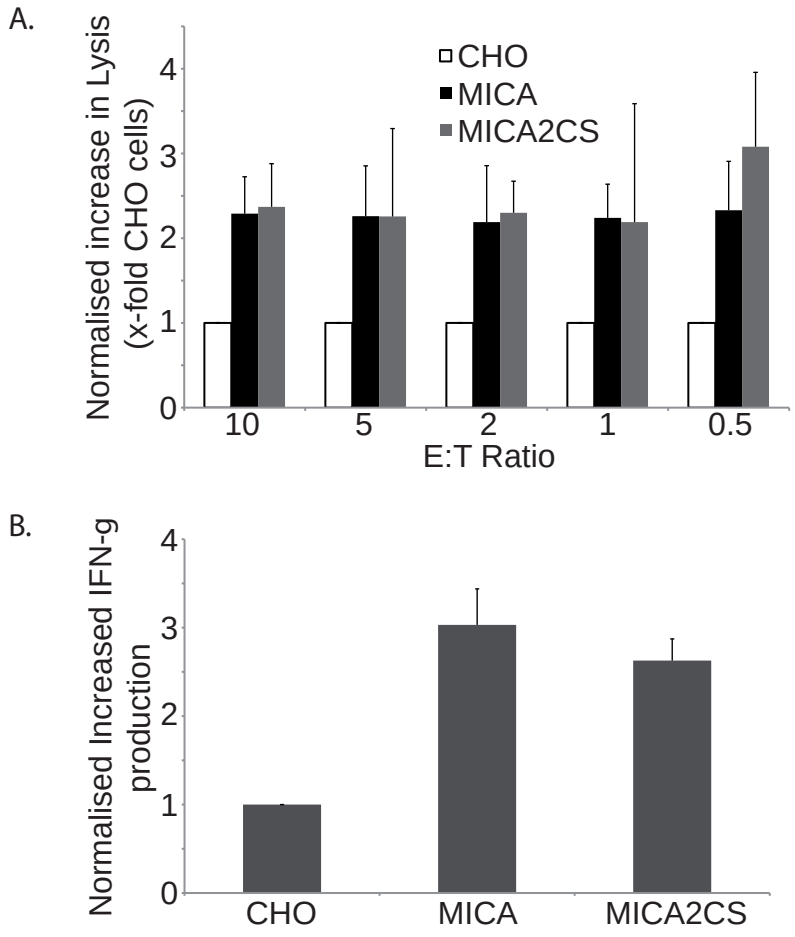
Supplementary Figure 1.

a. Expression of MICA by the glioma cell line U373. Untransfected U373 cells, U373/MICA and U373-MICA2CS transfectants were stained with either isotype control antibody or anti-MICA mAb. Samples were analysed by flow cytometry. **b- Shedding of the palmitoylation-deficient MICA2S molecules is also decreased in U373 cells.** Tissue culture supernatants from either U373-MICA or U373-MICA2CS cells, pre-incubated with the indicated inhibitors, were harvested at 8h and analysed by sandwich ELISA. In this panel, the background was subtracted.



Supplementary Figure 2.

a- Target cells expressing either wild type MICA or MICA2CS molecules are lysed equally by resting NK cells. Human NK cells, freshly isolated from peripheral blood, (resting NK cells) were used as effector cells in Europium-release killing assays (see methods). Untransfected CHO cells or CHO cells stably transfected with either MICA or MICA2CS were used as target cells. Targets were labelled with the fluorescent enhancing ligand DELFIA-BATDA (Perkin Elmer), washed and co-incubated with resting NK cells at the indicated E: T ratios during 2 h at 37°C. Supernatants were transferred to a europium solution and the lysis of target cells was measured by assaying the release of fluorescent ligand. The data presented are the average of five experiments using NK cells from different donors and have been normalised so that the data shown represent fold-increase in lysis of transfected compared to untransfected CHO cells. There are no statistically significant differences between NK lysis of CHO cells transfected with MICA or MICA2CS. **b- NK lysis of MICA and the palmitoylation mutant is not affected by the inhibition of metalloproteinases.** CHO-MICA or MICA2CS cells were cultured alone or with the inhibitor of metalloproteinases GM6001 (25 μM) for 2 hours at 37°C before using them as target cells in Europium-release killing assays performed as described above. The data shown are the average of three experiments with two donors and have been normalised as above. Treatment of target cells with metalloprotease inhibitors does not lead to statistically significant differences in NK lysis of CHO cells transfected with either MICA or MICA2CS.



Supplementary Figure 3.
MICA and the palmitoylation mutant MICA2CS are equally able to trigger cytotoxicity and cytokine production by IL-2 activated NK cells. a- CHO cells expressing either MICA or MICA2CS are equally efficient in triggering cytotoxicity by IL2-activated NK cells. IL2-activated NK cells were used 3 days post-IL2 stimulation in Europium-release killing experiments. The incubation time was 4 h at 37°C. The data shown are the average of three experiments with two donors and have been normalised as above. There are no statistically significant differences between activated NK cell lysis of CHO cells transfected with MICA or MICA2CS.
b- MICA and MICA2CS expressing CHO cells are equally efficient in triggering interferon-g **production by IL2-activated NK cells.** NK cells were cultured alone or co-cultured with the indicated target cells for 6hrs in the presence of Brefeldin A. Cells were then stained with FITC-labelled CD56, fixed with 4% paraformaldehyde, permeabilised by treatment with 0.1% Saponin and then stained with PE-labelled IFN-γ specific antibodies. The data shown are the average of three experiments with two donors and have been normalised so that the data shown represent fold-increase in interferon-γ production by NK cells incubated with transfected, compared to untransfected, CHO cells. There are no statistically significant differences in IFN-γ secretion between NK cells exposed to CHO cells transfected with either MICA or MICA2CS.